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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
C07H 1/08, C12Q 1/68, C12P 19/34	A1	WO 96/00228
		(43) International Publication Date: 4 January 1996 (04.01.96)

(21) International Application Number: PCT/US95/07940

(22) International Filing Date: 23 June 1995 (23.06.95)

(30) Priority Data:
08/264,556 23 June 1994 (23.06.94) US

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(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHOD FOR THE RAPID ISOLATION OF NUCLEIC ACID

(57) Abstract

A method is disclosed for isolation of DNA and substantially intact RNA from a biological sample. The invention comprises the steps of incubating the sample with a lysing buffer comprising an ionic detergent. The lysing buffer is substantially free of guanidine compounds. A salt solution is then added to the incubated mixture, and precipitated detergent-protein is separated from the nucleic acid in solution. A nucleic acid-precipitating agent is added to the nucleic acid solution, and the precipitated nucleic is recovered. Nucleic acid isolated according to the invention is suitable for cloning, construction of libraries and for use as a template in amplification reactions.

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Method for the rapid isolation of nocleic acid.

Field of the Invention

This invention relates to isolation of nucleic acids from biological samples. More particularly, this invention relates to methods and articles of manufacture useful for isolating RNA or DNA from biological samples in a simple, rapid manner and to the use of such isolated nucleic acid as a template in amplification reactions.

Background of the Invention

Procedures for the isolation of nucleic acids from biological samples are well-known in the art. Isolated nucleic acids are useful for many biotechnology-related purposes such as gene cloning, analysis of translation products in vitro and detection of particular sequences for diagnostic assays. Isolated nucleic acids often serve as templates for amplification protocols such as polymerase chain reaction (PCR), self-sustained sequence replication (3SR) and nucleic acid sequence-based amplification. Saiki et al., Science 239:487 (1988); Guatelli et al., Proc. Natl Acad. Sci. USA 87:1874 (1990); U.S. Patent No. 5,130,238 to Malek, L., et al.

Some methods have been developed to isolate a particular type of nucleic acid such as ribonucleic acid (RNA). One method for isolating RNA from biological samples is described by Chirgwin, J.M. et al., Biochemistry 18:5294 (1979). This method yields RNA that can be translated in an in vitro translation system and that is suitable for synthesis of cDNA. However, the method requires several hours to complete and involves several centrifugation steps.

Another procedure for isolating RNA is described in U.S. Patent 4,843,155 to Chomczynski, which procedure uses a composition comprising a guanidinium salt and acid phenol. U.S. Patent 4,935,342 to Seligson and Shrawder describes a method for isolating RNA by utilizing ion exchange columns. U.S. patent 5,155,018 to Gillespie and Cuddy describes a method for isolating RNA using siliceous material, such as finely-divided glass, in the presence of a concentrated, acidified chaotropic salt.

A particularly difficult aspect of RNA isolation is the presence of ribonucleases in many biological tissues and specimens. Ribonuclease activity during the isolation of RNA generally results in unwanted degradation of RNA species present in the sample. Ribonucleases are noted for their stability under conditions that denature most other proteins, and for the ease with which denatured ribonucleases may renature. A major concern in methods intended to isolate RNA is preventing ribonuclease activity sufficiently to allow intact RNA to be recovered.

Many methods have been described for isolation of deoxyribonucleic acid (DNA) from biological samples. One early procedure for isolating DNA from biological samples is described in Marmur, J., J. Mol. Biol. 3:208 (1961). This procedure yields DNA of relatively high purity, but is rather time-consuming. More rapid methods of DNA isolation, such as known methods for rapid isolation of plasmid DNA from bacteria, are described, for example, in Bimboim, H.C. and Doly, J., Nucl. Acids Res. 7:1513 (1979) and in Holmes, D.S. and Quigley, M., Anal. Biochem. 114:193 (1981). Other DNA isolation methods are disclosed in, for example, Vogelstein and Gillespie, Proc. Natl. Acad. Sci. USA 76:614 (1979) and Gross-Bellard et al. Eur. J. Biochemistry 36:32-38, (1973).

Certain methods are intended to isolate total nucleic acids, including both DNA and RNA. U.S. Patent 5,128,247 to Koller describes a method for isolating nucleic acids from cells using a chaotropic agent such as guanidinium isothiocyanate and subsequent exposure to a polyanion-containing agent such as heparin. This method is intended for isolation of high molecular weight nucleic acids for cloning. Another method for isolating RNA and DNA is disclosed in U.S. Patent 5,010,183 to Macfarlane, which describes a method using a cationic detergent.

Many of the known nucleic acid isolation methods suffer from one or more drawbacks. For example, some methods require several hours or days to complete. Certain methods require column separation protocols that use expensive solid phase supports. It is known that nucleic acids isolated by some methods are difficult to amplify by PCR or 3SR. Thus, there is a continuing need for methods suitable for preparing amplifiable nucleic acid templates. Further, there is a need for rapid, low cost methods for isolating nucleic acids, for example, for use in a clinical assay. A clinical work-up of a biological sample in which nucleic acid will be analyzed generally requires that nucleic acid be isolated, the assay protocol be performed, the results be interpreted and analytical reports be prepared. A rapid nucleic acid isolation method would assist in the completion of clinical assays in a short period of time, preferably within one day.

There also is a need for simple nucleic acid isolation methods that allow processing of multiple clinical specimens. Many known isolation methods require multiple steps and it would be desirable to have methods using fewer steps and having fewer components.

Summary of the Invention

A method is disclosed for isolating total nucleic acid from a biological sample comprising RNA, DNA and protein, in which the isolated RNA is substantially intact. The method comprises the steps of a) incubating the sample in a lysing buffer comprising an ionic detergent, for a time from less than about 1 minute to about 120 minutes; b) precipitating a major portion of the protein in the incubated sample by adding a salt composition under conditions where the nucleic acid remains substantially in solution; c) separating the precipitated protein from the nucleic acid in solution; d) precipitating the nucleic acid from the nucleic acid solution using a nucleic acid-precipitating agent; and e) recovering the precipitated nucleic acid. Notably, the lysing buffer is substantially free of guanidine compounds such as guanidine thiocyanate.

The lysing buffer also may include a proteinase or a ribonuclease inhibitor. The ribonuclease inhibitor may comprise vanadyl ribonucleoside complex.

The incubating step may occur for a time from less than about 1 minute to about 5 minutes and at a temperature from about 22°C to about 65°C. The method may further comprise the step of extracting residual protein from the nucleic acid solution after the separating step, using a protein-extracting agent.

Nucleic acid recovered according to the invention may be suitable for use as a template in an amplification reaction. For example, recovered RNA may be effective for cDNA synthesis and amplification by 3SR and recovered DNA may be effective for amplification by PCR.

An article of manufacture is disclosed, comprising packaging material and a lysing buffer within the packaging material. The lysing buffer comprises an ionic

detergent, and is substantially free of guanidine compounds. The article further comprises a label or package insert accompanying the packaging material, which indicates that the lysing buffer is suitable for isolating total nucleic acid according to a method of the invention. An article of manufacture may further comprise a salt composition, in which case the label or package insert further indicates that the salt composition can be used to isolate total nucleic acid according to a method of the invention.

Brief Description of the

Figure 1 is a photograph of an ethidium bromide-stained agarose gel showing the yield of nucleic acid isolated according to the invention.

Figure 2 is a photograph of an ethidium-bromide stained agarose gel. Lanes 1 and 6 are Lambda DNA digested with HinDIII. Lanes 2-5 show 1/20th of the nucleic acid isolated from various numbers of HeLa cells according to the invention. Lane 2, 1 x 10^5 cells; lane 3, 5 x 10^5 cells; lane 4, 1 x 10^6 cells; lane 5, 5 x 10^6 cells.

Figure 3 is an autoradiogram of a slot blot, showing 3SR reaction products prepared from template nucleic acid isolated in Figure 1. Slots Al-A3 show the products prepared from nucleic acid in lanes 2-4 of Figure 1, respectively. Slots Bl-B3 show the products prepared from nucleic acid in lanes 5-7 of Figure 1, respectively. Slots Cl-C3 show products prepared from nucleic acid in lanes 8-10 of Figure 1, respectively. Slot Dl shows the product prepared from nucleic acid in lane 12 of Figure 1. Slot El shows the product prepared from nucleic acid in lane 11 of Figure 1.

Figure 4 is a photograph of an ethidium bromide-stained agarose gel showing the yield of nucleic acid using the step of removing residual protein.

Figure 5 is a photograph of an ethidium bromide-stained agarose gel showing the nucleic acid yield using different combinations of salt solutions and nucleic acid-precipitating agents.

Figure 6 is an autoradiogram of a Northern blot of nucleic acid samples isolated according to the invention, using a probe complementary to the *bcr2-abl2*. Lanes 1-3: 1, 5, and 10 µl, respectively, of nucleic acid isolated using 1.0 µg/ml proteinase K in the lysing buffer; Lanes 4-6: 1, 5, and 10 µl of nucleic acid isolated without proteinase K in the lysing buffer.

Figure 7 is a photograph of an ethidium bromide-stained agarose gel, showing amplification of nucleic acid isolated according to the invention. Lane 1: ØX174 digested with HaeIII; Lanes 2-4: 1, 5, and 10 µl, respectively, of nucleic acid isolated from SiHa cells and amplified by PCR using primers MY11 and MY09; Lane 5: Control reaction mixture using MY11 and MY09 primers without added template; Lanes 6-7: 2 and 7 µl, respectively, of nucleic acid from K562 cells converted to first strand cDNA and amplified by PCR using primers BB164 and BB165; Lane 8: 7 µl of nucleic acid from K562 cells amplified by PCR using primers BB164 and BB165 without conversion to cDNA; Lanes 9-10: 2 and 7 µl respectively of nucleic acid from K562 cells converted to first strand cDNA and amplified by PCR using primers BB160 and BB165; Lane 11: 7 µl of nucleic acid from K562 cells amplified by PCR using primers BB160 and BB165 without conversion to cDNA; Lane 12: 7 µl of nucleic acid from K562 cells isolated by RNAzol B, converted to first strand cDNA and amplified by PCR using primers BB164

and BB165; Lane 13: 7 ytl of nucleic acid from K562 cells isolated by RNAzol B, converted to first strand cDNA and amplified by PCR using primers BB160 and BB165; Lane 14: ØX174 digested with HaeIII.

Figure 8 is a photograph of an ethidium-bromide stained agarose gel. Lanes 1 and 6 are ØX174 DNA digested with HaeIII. Lanes 2-5 show different dilutions of the PCR product from nucleic acid isolated from HeLa cells using the MY09/MY11 HPV consensus primers. Lane 2, 1:1000 dilution; lane 3, 1:750 dilution; lane 4, 1:500 dilution; lane 5, 1:250 dilution.

Figure 9 is a photograph of an ethidium bromide-stained agarose gel, showing the PCR products of nucleic aid isolated from cervical swabs. The primers used in Row 1 were MY09/MY11 and the primers used in Row 2 were GH20/PCO4. Samples treated only with proteinase: lanes 2, 4, 6, 8 and 10 from samples 1, 2, 3, 4 and 5, respectively. Samples treated according to the invention: lanes 3, 5, 7, 9 and I 1 from samples 1, 2, 3, 4 and 5, respectively.

Detailed Description of the Invention

The applicant has discovered methods that result in rapid isolation of total nucleic acid from a biological sample, while utilizing few solution components and utilizing inexpensive equipment. Methods of theinvention involve lysing a biological sample in a lysing buffer, which solubilizes many cellular proteins and frees nuclear proteins from DNA and RNA, followed by separation of nucleic acid from protein and recovery of the isolated nucleic acid. Surprisingly, RNA present in the isolated nucleic acid is substantially intact. Isolated DNA or RNA can be used as a template in an

amplification reaction. In particular, isolated nucleic acid is useful as a template for 3SR or PCR. Further, RNA isolated by the method of the invention is suitable for cloning of mRNA species. An advantage of the present invention is the rapidity with which nucleic acid may be isolated from a biological sample. The method of the invention shortens considerably the time required for completing an amplification-based diagnostic analysis of a biological specimen, since the lysis of the biological sample may be completed in less than about 1 minute.

A biological sample may comprise intact cells, clumps of cells, portions of cells, isolated nuclei, or tissue. A biological sample may be preserved, e.g., fixed, frozen and/or embedded, provided that such preservation permits subsequent isolation of substantially intact RNA and DNA. For example, a biological sample may be derived from a specimen that has been preserved for archival purposes, e.g., fixed and embedded in paraffin. A biological sample may be fixed and/or frozen to allow later analysis, e.g., at a laboratory distant from the clinic where the sample was taken or when a sufficient number of samples have been accumulated for efficient isolation of nucleic acid. Altematively, a biological sample may be a fresh preparation, i.e, a specimen that is to have nucleic acid isolated within a short period of time after the sample is taken, without an intervening preservation step.

A biological sample may be from a source such as animal tissue or cells, including without limitation a cell culture, intact tissue pieces (e.g., a biopsy), or a whole organ. Alternatively, a biological sample may comprise plant or fungal tissue or cells, which may be processed before performing a method of the invention, for example, by removing the cell walls. The amount of tissue or the number of cells present in a

biological sample may be adjusted as desired, e.g., to achieve a desired yield of nucleic acid. For example, if cultured mammalian cells are the source of a biological sample and the isolated nucleic acid is to be a template for an amplification reaction, a suitable amount may be from about 500 to about 1 X 10^7 cells.

A biological sample is incubated in a lysing buffer, which comprises an ionic detergent. An ionic detergent such as sodium dodecyl sulfate (SDS) is thought to solubilize and to quantitatively bind to proteins in the sample, allowing protein-detergent complexes to be precipitated when the salt concentration of the mixture is increased in the second step of the method. The concentration of ionic detergent in a lysing buffer is generally from about 0.05% to about 5%, preferably from about 0.1% to about 2%, more preferably from about 0.3% to about 1.5%.

Additional, optional components may be included in a lysing buffer if desired. For example, lysing buffer may comprise an ionic detergent, a ribonuclease inhibitor, a sulffiydryl reducing agent and a pH buffering agent. When isolation of substantially intact DNA is desired, lysing buffer may comprise an ionic detergent and a pH buffering agent. However, additional components such as a sulffiydryl reducing agent and a ribonuclease inhibitor do not interfere with isolation of DNA.

Ribonuclease inhibitors are optionally included in a lysing buffer to reduce degradation of RNA in the biological sample during the isolation procedure. An illustrative example of a ribonuclease inhibitor is vanadyl-ribonucleoside complex (VRC). Vanadyl-ribonucleoside complex comprises a mixture of the complexes formed between an oxovanadium IV ion and each of the four ribonucleosides. These complexes are transition-state analogs that bind to many RNases and nearly completely inhibit

enzyme activity. Berger, S. and Birkenmeier, C., Biochemistry 18:5143 (1979). Other suitableribonuclease inhibitors include malacoid and bentonite.

A sulffiydryl reducing agent optionally is included in the lysing buffer. Illustrative examples of such reducing agents are dithiothreitol and ß-mercaptoethanol. A pH buffering agent is optionally included to ensure that no unwanted changes occur in the pH of the lysing buffer. It is known that highly alkaline solutions can degrade RNA and that highly acid solutions can precipitate and depurinate nucleic acids. Typical pH buffering agents include Tris (hydroxymethyl)-aminomethane, sodium or potassium phosphate and morpholino-propane sulfonic acid.

Lysing buffer optionally contains a proteinase, which may assist in freeing nuclear proteins from the nucleic acids. Lysing buffer preferably contains a proteinase if a biological sample comprises cell clumps or intact tissue pieces. Inclusion of a proteinase is particularly preferred when the resulting nucleic acid is to be used as a template in an amplification reaction. Illustrative examples of proteinases are proteinase K, Pronase™ (self digested), and pepsin. Such proteinases typically are present in a lysing buffer from about 1 to about 500 µg/ml.

A lysing buffer according to the invention is substantially free of guanidine compounds, which are potent irritants. Many previously known lysing buffers used in RNA isolation have guanidine compounds as a component. For example, the lysing buffers in U.S. Patent 4,843,155 and the RNAgents® Total RNA Isolation System, Catalog number Z5110, Promega Corporation, Madison Wl 53711-5399, have guanidine thiocyanate as a component. As used herein, the term guanidine compounds refers to

guanidine and guanidinium compounds and salts thereof, including, but not limited to guanidine thiocyanate and guanidinium chloride.

The length of time allotted for incubation of a biological sample in lysing buffer depends to some extent upon the nature of the biological material, e.g., species, cell type, amount of sample and the like. Biological samples such as cultured mammalian cells generally require from less than about 1 to about 120 minutes, preferably about 1 to about 60 minutes, more preferably about 1 to about 30 minutes. Most biological samples will require less than about 60 minutes. The amount of lysing buffer added to a sample is sufficient to allow separation of protein and recovery of nucleic acids in subsequent steps. The amount of lysing buffer is adjusted according to factors such as species and tissue type of the sample, the amount of sample and the like. It will be apparent to those skilled in the art that it may be useful to agitate the mixture before or during the incubation in order to facilitate disruption of the sample, e.g., shaking, vortexing, or equivalent agitation method.

A solution comprising a major salt and a minor salt is then added to the incubated mixture. A major salt may be, for example, potassium acetate, potassium chloride, sodium acetate or sodium chloride. A minor salt may be a salt such as magnesium chloride or sodium chloride. When SDS is the ionic detergent, potassium and/or magnesium ions are preferably used to form the salt composition since sodium salts are not as effective at precipitating SDS.

The amount of salt composition added to the incubated mixture is sufficient to cause detergent-protein complexes to precipitate, e.g., in about 10X to about 100X molar excess of the major salt compound to the ionic detergent in the lysing buffer. For

example, when an equal volume of a salt composition is added to an incubated mixture comprising 0.5% to 2.0% SDS, the concentration of the major salt is generally from about 0.8 M to about 2.5 M, preferably from about 1.0 M to about 1.8 M. The concentration of the minor salt is generally from about 25 mM to about 75 mM, preferably from about 40 mM to about 60 mM.

After addition of the salt composition, precipitation of detergent-protein complexes may be accelerated, if desired, by chilling the mixture on ice for a brief period of time, typically about 1-5 minutes. It is not necessary in most cases to chill the mixture for more than about 10 minutes.

Precipitated detergent-protein is separated from the supematant, which contains most of the nucleic acid, by any suitable means. For example, detergent-protein may be separated by centrifugation of the mixture or by adding a protein-binding resin such as Strataclean™ resin (Stratagene, La Jolla, California, 92037), Pro-Cipitate™ (Affinity Technology, Inc. New Brunswick, NY 08901) or other commercially available protein removal matrix. Alternatively, detergent-protein may be separated by adding phenol and centrifuging the mixture. If phenol is used in removing detergent-protein, it is preferable to use phenol with a pH of about 7.5 or above when isolating total nucleic acids or a pH of about 6.0 or below when preferentially isolating RNA, preferably a pH of about 4.5.

After separation of the precipitated detergent-protein, the supernatant may contain a residual amount of protein in addition to nucleic acids. It may be desirable for some applications to remove residual protein, which can be accomplished using an additional protein extraction step. Phenol is a known protein-extracting agent that is suitable for removing residual protein, typically by addition of an equal volume of

water-saturated phenol to the supernatant. Other protein-extracting agents may be used if desired.

The use of phenol containing 8-hydroxyquinolone (e.g., at about 0.1%) is preferred when ribonuclease inhibitors such as vanadyl-ribonucleoside complex are present in lysing buffer, since 8-hydroxyquinolone will extract these inhibitors and phenol will extract protein. Multiple extractions with phenol containing 8-hydroxyquinoline may be performed, if desired.

Total nucleic acid is precipitated from the supernatant (after extraction of residual protein, if desired) by the addition of a nucleic acid-precipitating agent. Suitable agents include 2 volumes of ethanol, 1/2 to 1 volume of isopropanol, or 1 volume of a solution comprising 5% w/v cetyltrimethylammonium bromide (CTAB).

The precipitated nucleic acid is recovered by means appropriate to the end use of the recovered nucleic acid. For example, nucleic acids may be recovered by centrifuging the mixture in order to pellet the nucleic acid precipitate. Alternatively, nucleic acids may be recovered by filtration on nitrocellulose or glass fiber filters. Nucleic acids are preferably recovered by centrifugation and the resulting pellet is then resuspended in an aqueous solution.

The recovered nucleic acid is useful for a number of end uses. The nucleic acid may be treated with RNase-free DNase and used to prepare cDNA libraries or to clone specific mRNAs. The nucleic acid may be used to prepare genomic libraries or to clone specific genes.

A particularly preferred use is as a template in an amplification protocol such as 3SR or PCR. It is known that clinical samples may be difficult to prepare for use in a

nucleic acid amplification protocol. For example, an ongoing problem with diagnostic assays for human papillomavirus (HPV) in cervical swab samples is the inability to amplify target DNA by PCR. Since small amounts of clinical material may be available for analysis, the amount of HPV-containing target DNA may be quite small. The present invention overcomes such difficulties, since nucleic acid isolated from clinical samples according to the invention is readily amplifiable.

The invention will be understood with reference to the following illustrative embodiments, which are purely exemplary, and should not be taken as limiting the true scope of the present invention as described in the claims.

EXAMPLE 1.

Isolation of Substantially Intact RNA and DNA from K562 cells.

A sample of human K562 cultured cells was obtained from American Type Culture Collection (Rockville, Maryland). K562 is a line of pleuroeffusion cells derived from a 53 year old female suffering from chronic myelogenous leukemia (CML) in blast crisis. The cells were grown in RPMI with 10% fetal bovine serum and maintained by standard culture methods. MDCK cells (a canine kidney cell culture) were also obtained from the American Type Culture Collection and were grown and maintained as described for K562 cells.

Approximately 2 X 10^6 MDCK cells and I X 10^4 K562 cells were added as a mixed preparation to each sample tube. Cells in each tube were mixed with 450 µl of lysing buffer and the mixture incubated at 50°C. Lysing buffer contained 0.5% SDS, 0.1

M dithiothreitol and 50 mM Tris HCL, pH 7.4. Vanadyl-ribonucleoside complex and proteinase K were included in the lysing buffer as indicated in Table 1. Lysing buffer was stored at room temperature and was stable for about 14 days.

Table 1.
Lysing Buffer Compositions
Used to Isolate the Nucleic Acid Samples of Figure 1

Lane[c]	VRC (mM)	Protein-ase K (µ/MI)	Incubation Time[a] (minutes)
2	1	0.5	10
3	1	0.5	20
4	1	0.5	30
5	5	0.5	10
6	5	0.5	20
7	5	0.5	30
8	1	0	30
9	5	0	30
10	0	0.5	30
11	0	1.0	30
12	RNAzol B[b]		

[a] Incubation time in the indicated lysing buffer.
[b] RNAzol™ (Teltest, Friendswood, TX) was used to isolate nucleic acid according to manufacturer's directions
[c] Corresponding lane in Figure 1

An equal volume of precipitating salt solution, containing 1.6 M potassium acetate and 50 mM magnesium chloride, was added to the lysed sample. This mixture was incubated for 5 minutes at 4°C, precipitating most of the protein in the sample. The mixture was centrifuged at 4000 RPM for 5 minutes to separate the precipitated protein from the nucleic acid in the supernatant. Nucleic acid in the supernatant was then precipitated by the addition of an equal volume of isopropanol. The nucleic acid precipitate was collected by centrifugation. The nucleic acid pellet was recovered by

resuspending in 50 µl of 10 mM Tris, 1 mM EDTA (TE), pH 7.4. A 5 µl aliquot of the recovered nucleic acid was loaded on a 0.8% agarose gel which included 1 µl/ml ethidium bromide and electrophoresed at 80 V for 1 hour. The results are shown in Figure 1. HaeIII-digested ØX174 and Hind III-digested λ DNA were used as molecular weight markers (Lanes 1 and 13).

As shown in Figure 1, incubating the cells in lysing buffer for longer than about 10 minutes does not appear to significantly increase the amount of nucleic acid recovered by the method of the invention. A visually observable amount of nucleic acid was recovered in the absence of a proteinase in the lysing buffer, as illustrated in lanes 8 and 9.

EXAMPLE 2.

Isolation of Substantially Intact RNA and DNA from HeLa cells.

HeLa cells were added to 400 µl of 0.5% SDS and an equal volume (400 µl) of 1.6M KCl, 5OmM $MgCl_2$ was added to the sample immediately thereafter. The mixture was placed on ice for 5 minutes, 800 µl of phenol:chloroform:isoamyl alcohol was added and the mixture was immediately vortexed for 10 seconds. The resulting suspension was centrifuged at 6,000 rpm for 5 min. The aqueous layer was collected and 800 µl of isopropanol was mixed into the mixture. The precipitated nucleic acid was recovered by centrifugation at 14,000 rpm for 15 min. The nucleic acid pellet was washed once with 70% ethanol and the pellet was resuspended in 50 µl TE, pH 7.4. Figure 2 shows 1/20th

of the resuspended nucleic acid after electrophoresis on a 0.8% agarose gel containing ethidium bromide.

As illustrated in Figure 2, DNA and ribosomal RNA in the isolated nucleic acid was intact and migrated as discrete bands.

EXAMPLE 3.

Amplification of Isolated RNA by 3SR.

This example teaches that RNA recovered in accordance with the invention can be used as a template in 3SR amplification.

A 5 µl aliquot of each nucleic acid sample of Example 1 was used as a template in a 3SR amplification reaction in an RNase-free 1.5 ml Eppendorf tube. The reaction mixture contained 20 µl of a 5X buffer (containing 200 mM Tris HCI, pH 8.1,150 mM $MgCl_2$, 100 mM KCl, 50 mM dithiothreitol, 20 mM spermidine), 5 µl (15 pmol) of each of the priming oligonucleotides, 20 µl of a 5X nucleoside triphosphate mix (35 mM rNTP's, 5 mM dNTP's), and 45 µl of DEPC-treated H_2O. The sequences of the primers used in the reaction (325 and 329) are shown in Table 2. Each mixture was heat denatured at 65°C for 1 minute. Following denaturation, each tube was transferred to a 42°C water bath and incubated for 5 minutes. Thirty units of AMV reverse transcriptase, 2 units of E. coli Ribonuclease H and 1000 units of T7 RNA polymerase were added to each tube and the mixtures were incubated at 42°C for 60 minutes.

Table 2.
Oligonucleotides

SEQ. ID No.	Primer	Nucleotide Sequence
1	325	AATTTAATAC GACTCACTAT AGGGAAGATG CTGACCAACT CGTGTGT
2	329	TGCAACGAAA AGGTTGGGGT
3	BB302	GCTGAAGGGC TTTTGAACTC TGCTTA

An aliquot of each reaction, representing 1/10 of the total volume, was denatured in 90 µl of 7.4% formaldehyde and 10X SSC (Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, Vol. 2, 2nd edition, Cold Spring Harbor Laboratory Press, New York, 1989) at 65°C in a water bath for 10 minutes. Aliquots were ice-chilled immediately and loaded onto a nitrocellulose membrane through a slot-blot apparatus. Nucleic acid in the aliquots was immobilized on the nitrocellulose by baking at 80°C.

The filters were prewetted with hybridization solution (6X SSC, 10X Denhardt's solution, 10 mM Tris pH 7.4, 0.2 mg/ml sheared salmon sperm DNA and 1% SDS, then hybridized at 55°C for 45 minutes with a ^{32}P-labeled oligonucleotide probe (SEQ. ID No.: 3) that was complementary to the junction sequence of the *bcr2-abl2* translocation characteristic of chronic myelogenous leukemia in humans. Shtivelman, et al., Nature, 315: 550-554 (1985). After hybridization, the filters were washed three times at room temperature for 5 minutes each using 1 ml buffer/cm^2 filter, 2X SSC, 0.1% SDS. The filters were exposed to X-ray film at -70°C with one intensifying screen. The results of the 3SR amplification are shown in Figure 3.

As shown in the slot blot of Figure 3, nucleic acid isolated according to Example 1 was a suitable template for 3SR amplification. Those samples that had proteinase K

included in the lysing buffer appeared to yield substantially more amplification reaction product. The yield of amplified product was approximately the same at all incubation times in lysing buffer with proteinase K.

EXAMPLE 4.

Removal of Residual Protein.

This example teaches the removal of residual protein that maybe present after precipitation of the major portion of protein in the biological sample.

K562 cells and MDCK cells were collected and mixed in the proportions described in Example 1. Nucleic acid was isolated as described in Example 1, except that after separating the precipitated protein, residual protein was removed from the nucleic acid supernatant by various procedures. For one sample, 50 µl of Strataclean™ protein removal resin (Stratagene, La Jolla, California) were added to the supernatant. The mixture was vortexed and incubated for I minute and the resin was removed by centrifugation (Figure 4, lane **2**). For three other samples, 450, 125 or 250 µl of Pro-Cipitate™ (Affinity Technology, Inc., New Brunswick, NJ 08901) were added to the supernatant (Figure 4, lanes 3, 4 and 5, respectively) and the mixture was vortexed. After incubating for 1 minute at room temperature, the supernatant was centrifuged to remove the Strataclean™ matrix. After removal of the protein-extracting agent, nucleic acids in the supernatant were precipitated with an equal volume of isopropanol and resuspended in 50 µl of TE, pH 7.4. A 5 µl aliquot was loaded on an 0.8% agarose gel and electrophoresed for 1 hour at 80 V. A Hind Ill digest of λ DNA was used as a size marker (Figure 4, lane 1). As shown in Figure 4, the amount of nucleic acid recovered is easily visible in an ethidium bromide-stained gel for all four samples.

EXAMPLE 5.

Precipitation of Nucleic Acid

This example teaches that various salt compositions and nucleic acid-precipitating agents may be used in accordance with the invention.

K562 cells and MDCK cells were collected and mixed in the proportions described in Example 1. Samples were solubilized by vortexing at room temperature in 450 µl lysing buffer comprising of 0.5% SDS, 50 mM Tris pH 7.4, 0.1 M dithiothreitol, and 5 mM vanadyl ribonucleoside complex. An equal volume of the salt solution indicated in Table 3 was added to each sample and detergent-protein complexes removed by centrifugation. The supernatant was precipitated with the nucleic acid-precipitating agent indicated in Table 3. Protein was extracted from one sample using Stratacleanᵀᴹ resin as described in Example 3.

The precipitated nucleic acid was resuspended in 50 µl of TE, pH 7.4, and a 5 µl aliquot of each sample was electrophoresed in a 0.8% agarose gel containing 1 µg/ml of ethidium bromide. Figure 5 shows a photograph of the gel; the lane numbers correspond to those shown in Table 3. Lane 1 contains Hind]III-digested λ DNA. The results indicate that nucleic acid-precipitating agents such as isopropanol and CTAB are effective with either a KCl or a KOAc salt solution.

Table 3.
Nucleic Acid Isolation Solutions

Lane	Salt Composition	Protein Estracting Agent	Nucleic Acid Precipitating Agent	Centrifugation (RPM;Time)[h]
2	KCl[b]	--[d]	Isopropanol[f]	12,000; 5 min.
3	KCl	--	Isopropanol	4,0%; 5 min.
4	KOAc[c]	--	CTAB	4,000; 5 min.
5	KOAC	--	Isopropanol	12,000; 5 min.
6	KOAC	--	Isopropanol	4,000; 5 min.
7	KCl	--	CTAB	4,000; 5 min.
8	KCl	Strataclean[e]	Isopropanol	12,000; 5 min.
9	RNAzol	Strataclean	Isopropanol	12,000; 15 min.

[b] KCl = 1.6 M KCl, 50 mM $MgCl_2$ used as precipitating salt solution.
[c] KOAc = 1.6 M potassium acetate, 50 mM $MgCl_2$ used as precipitating salt solution.
[d] -- = No protein extracting agent used.
[e] Strataclean™ resin used as described in Example 3 to remove residual protein.
[f] One-half volume (450 µl) isopropanol used as nucleic acid-precipitating agent.
[g] Equal volume of 5% w/v CTAB, 50 mM Tris, pH 8.0,20 mM EDTA used as nucleic acid-precipitating agent.
[h] Centrifugation for indicated RPM and time at 4°C to collect nucleic acid precipitate.

EXAMPLE 6.

Demonstration of Substantially Intact RNA in Isolated Nucleic Acid.

K562 cells contain the *bcr2-abl2* and the *bcr3-abl2* translocations of chromosomes 22 and 9. Shtivelman, et al., Nature, 315:550-554 (1985). Nucleotide sequences for *bcr* and *abl* are in Genbank Accession Nos. 74469 and 81414, respectively. The messenger RNA produced by the *bcr-abl* translocation is about 8 kilobases (kb) in length. A Northern blot was performed to determine the size of *bcr-abl* mRNA in nucleic acid isolated according to the invention. Total nucleic acid was isolated from K562 suspension cells by solubilizing 1 X 10^6 cells in 450 µl of lysing buffer containing 0.5% SDS, 50 mM Tris pH 8.0, 0.1 M dithiothreitol, and 5 mM

vanadyl ribonucleoside complex. An SDS-protein complex was formed by adding 450 µl of 1.6 M KCl, 50 mM $MgCl_2$ to the mixture, vortexing briefly and incubating on ice for 5 minutes. Approximately 900 µl of Tris-saturated phenol containing 0.1% 8-hydroxyquinoline, pH 8.0, was added to the mixture. After vortexing, the mixture was centrifuged for 10 minutes at 12,000 RPM and the supernatant was collected. Nucleic acid was precipitated from the supernatant using an equal volume (900 µl) of isopropanol and pelleted by centrifugation at 12,000 RPM for 10 minutes. The pellet was resuspended in 20 µl TE, pH 7.4. One, 5, and 10 µl of the resuspended nucleic acid was loaded onto a 1.0% formaldehyde gel and electrophoresed at 50V for 1.5 hours. The gel was then soaked in DEPC-treated water for 45 minutes and in 20X SSC for 45 minutes and nucleic acids transferred by capillary transfer from the gel onto a nitrocellulose membrane. After transfer, the nitrocellulose was prewetted with hybridization solution (6X SSC, 10X Denhardt's solution, 10 mM Tris, pH 7.4, 0.2 mg/ml sheared salmon sperm DNA and 1.0 % SDS), hybridized with a ^{32}P-labeled oligonucleotide (SEQ. ID No.: 3) complementary to the junction sequence of *bcr2-abl2* and exposed to X-ray film. An autoradiogram of the blot (Figure 6) showed that the probe hybridized to a band corresponding to the size expected for *bcr-abl* mRNA. This result indicates that substantially intact mRNA was recovered from the isolated nucleic acid.

EXAMPLE 7.

Amplification of Isolated RNA by PCR.

This example teaches that RNA recovered in accordance with the invention can be used to generate a template suitable for PCR amplification.

Nucleic acid was isolated as described in Example 6 from K562 cells. The precipitated nucleic acid was resuspended in 50 µl of TE, pH 7.4, and an aliquot was used to synthesize first strand DNA. The reaction conditions used to synthesize first strand cDNA were: 3 µl 0.1M dithiothreitol, 50 pmol random hexamer, 4.0 µl of dNTP (2.5 mM each nucleotide), 3.0 µl 5X buffer (250 mM Tris pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1.0 µl RNAsin (40 units/µl), 1.0 µl superscript reverse transcriptase (200 units/µl BRL, Bethesda, MD), either 2 or 7 µl of resuspended nucleic acid and diet.hylpyrocarbonate (DEPC)-treated water to a final volume of 30 µl. The reaction was incubated for 30 minutes at 37°C.

One half of the first strand reaction mixture was used in PCR. Two pairs of PCR primers were used, BB164/BB165 and BB160/BB165. The PCR reaction contained 10 µl dNTP (2.5 mM each nucleotide), 10 µl 10X PCR buffer (100mM Tris pH 8.0, 500 mM KCl), 2.5 µl 50 mM $MgCl_2$, 50 pmol each priming oligonucleotide, and water to bring the final volume to 99 µl. Each PCR mixture was incubated for 7 minutes at 95°C; amplification was initiated by the addition of 1 µl of Taq DNA polymerase. The thermocycle conditions were 35 cycles of 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C. The final extension was completed by incubating the mixture for 7 minutes at 72°C.

The PCR reaction products were electrophoresed on a 1.5 % agarose gel containing 1 µl/ml of ethidium bromide at 100 V for 1 hour. A 489 bp fragment was the expected product from primers BB164 and BB165. This fragment migrates on a 1.5% gel at approximately at 600 bp, as is known in the art. Such a fragment is visible in Figure 7, lanes 6-7. A 783 bp band corresponding to the reaction product expected from primers BB160 and BB165 is clearly visible in Figure 7, lanes 9-10. For both primer pairs, the reaction product from template nucleic acid isolated according to the invention migrates at the same position as a positive control, as shown in lanes 12-13. No reaction product is visible in control reactions in which RNA was not converted to cDNA, as shown in lanes 8 and 11. These results indicate that RNA isolated according to the invention is successfully converted to DNA suitable as a template for PCR.

Table 4.
Oligonucleotides

Seq. ID No.	Primer	Nucleotide Sequence
4	BB160	GACTGCAAAT GGTACATTCC G
5	BB164	TCTGACTATG AGCGTGCAGA G
6	BB165	ACTGCTCTCA CTCTCACGCA
7	MY09	CGTCCMARRG GAWACTGATC
8	MY11	GCMCAGGGWC ATAAYAATGG
9	GH20	GAAGAGCCAA GGACAGGTAC
10	PCO4	CAACTTCATC CACGTTCACC

EXAMPLE 8.

Amplification of Isolated DNA by PCR.

This example teaches that DNA recovered in accordance with the invention can be used as a template in PCR amplification.

Nucleic acid was isolated from SiHa cells as described in Example 6. The precipitated nucleic acid was resuspended in 50 µl of TE, pH 7.4, and 1, 5, and 10 µl of the nucleic acid was used as a template for PCR. The PCR reaction contained were 10 µl dNTP (2.5 mM each nucleotide), 10 µl 10X PCR buffer (100 mM Tris pH 8.0, 500 mM KCl,), 2.5 µl 50 mM $MgCl_2$, 50 pmol of each primer, water to a final volume of 99 µl. The PCR mixture was incubated for 7 minutes at 95°C and amplification initiated by the addition of 1 µl of Taq DNA polymerase. The thermocycle conditions were the same as the conditions used in Example 7. The primers used were MY09 and MY11. MY09/MY11 amplifies a 449-458 base pair portion of the human papillomavirus (HPV) Ll coat gene of genital HPV strains. The PCR reaction product was electrophoresed at 100 V for 1 hour on a 1.5 % agarose gel containing 1 ytg/ml of ethidium bromide. As shown in Figure 7, lanes 2-4, a 452 bp band was clearly visible. No reaction products were visible in a control reaction that lacked a template (Figure 7, lane 5).

In a separate experiment, nucleic acid isolated from HeLa cells in Example 2 was diluted and subjected to PCR as described above. Figure 8 is a photograph of a 1.5% agarose/ethidium bromide gel of the PCR product. A fragment of the size expected for this portion of the HPV L1 coat gene is visible.

EXAMPLE 9.

Processing of Clinical Samples

Cervical swab samples were obtained from a referral clinic in Boston, MA. Samples (100 µl) were incubated in 100 µl of 400 µg/ml proteinase K, 1% SDS, 25 mM Tris-HC1, pH 8.5, and 0.5mM EDTA for 1 hour at 55°C. The proteinase K was

denatured at 95°C for 10 minutes and a 10 µl aliquot of each sample was used as a template for PCR. The primers used were MY09/MY11 and GH20/PCO4. GH20/PCO4 amplifies a 296 bp region of the human ß-globin gene and was used as a positive control for the ability of the sample to serve as a template.

Out of fifteen abnormal clinical cervical samples, only two were weakly positive by PCR for the presence of HPV. The amplification of ß-globin by GH20/PC04 was successful in 9 out of the 15 samples, but should have exceeded 90% (14 out of 15 samples). Samples that cannot be amplified in a positive control reaction are considered to be unsatisfactory for diagnostic assays that involve an amplification step.

An attempt was made to recover DNA in the processed samples by precipitating one-half of the proteinase-digested mixture with ammonium acetate/ethanol. The precipitated material was used as a template for PCR; the attempt was unsuccessful, in that no additional samples could be amplified by the ß-globin primers.

EXAMPLE 10.

Amplification of Nucleic Acid Isolated from Clinical Samples

An equal volume of 1.0% SDS was added to one-half (200 µl) of the digested clinical samples of Example 9. Four hundred µl of 1.6 M KCl, 50 mM $MgCl_2$ was added next and the mixture was set on ice for 5 min. An equal volume (800 µl) of phenol:chloroform:isoamyl alcohol was added to each mixture and vortexed for 10 seconds. Each mixture was centrifuged at 6,000 rpm for 5 min. The aqueous layer was collected and an equal volume (800 µl) of isopropanol was mixed into the material. Nucleic acid was recovered by centrifugation at 14,000 rpm in a microfuge for 15 min.

The visible pellet was allowed to dry at room temperature for one-half hour, and subsequently resuspended in 100 µl autoclaved distilled H_2O. A 10 µl aliquot of each sample, as well as a 10 µl aliquot of same samples digested as described in Example 9, was subjected to PCR. PCR was carried out as described in Example 7, using the MY09/MY11 primer pair and the GH20/PC04 primer pair. Aliquots of the PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide for 45 minutes. The results for 5 of the samples are presented in Figure 9.

As shown in Figure 9, no products are visible in the proteinase-treated samples, regardless of which primer set was used (Rows 1 and 2, lanes 2, 4, 6, 8 and 10). When nucleic acid was isolated according to the invention, a ß-globin PCR product was visible in all samples (Figure 9, Row 2, lanes 3, 5, 7, 9 and 11). An I-IPV PCR product was visible in three samples (Row 1, lane 3, 7 and 11). By isolating nucleic acid according to the invention, a significantly higher percentage of HPV-positive cervical specimens can be detected, compared to known isolation methods.

The foregoing detailed description has been provided for a better understanding only and no unnecessary limitation should be understood therefrom as some modifications will be apparent to those skilled in the art without deviating from the spirit and scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Janice T. Brown

(ii) TITLE OF INVENTION: RAPID ISOLATION OF NUCLEIC ACID

(iii) NUMBER OF SEQUENCES:10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Dade International Inc.
(B) STREET: 1717 Deerfield Rd., Box 778
(C) CITY: Deerfield
(D) STATE: Illinois
(E) COUNTRY: USA
(F) ZIP: 60015-0778

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM PC Compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: MS Word for Windows 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA

(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Louise S. Pearson
(B) REGISTRATION NUMBER: 32,369
(C) REFERENCE/DOCKET NUMBER: BA1-4646

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 708/267-5373
(B) TELEFAX: 708/267-5376
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: synthetic

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(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: synthetic

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(A) LENGTH: 26
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: synthetic

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Shtivelman, E. et al.
(B) TITLE:
(C) JOURNAL:Nature
(D) VOLUME: 315
(E) ISSUE:
(F) PAGES: 550-554
(G) DATE: 1985

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(A) LENGTH: 2
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: synthetic

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(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: synthetic

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(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: synthetic

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Manos, M.
Ting, Y.
Wright, D.
Lewis,
Broker, T.
Wolinsky, S.
(B) TITLE:
(C) JOURNAL: Cancer Cells
(D) VOLUME: 7
(E) ISSUE:
(F) PAGES: 209-214
(G) DATE: 1989

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Durst, M.
Gissman, L.
Ikenberg, H.
zur Hausen, M.
(B) TITLE:
(C) JOURNAL: Proc. Natl. Acad. Sci. USA
(D) VOLUME: 80
(E) ISSUE:
(F) PAGES: 3812-3815
(G) DATE: 1983

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CGTCCMARRG GAWACTGATC 20

(2) INFORMATION FOR SEQ ID NO: 8:

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(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: synthetic

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Manos, M.
Ting, Y.
Wright, D.
Lewis,
Broker, T.
Wolinsky, S.
(B) TITLE:
(C) JOURNAL: Cancer Cells
(D) VOLUME: 7
(E) ISSUE:
(F) PAGES: 209-214
(G) DATE: 1989

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(A) AUTHORS: Durst, M.
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(B) TITLE:
(C) JOURNAL: Proc. Natl. Acad. Sci. USA
(D) VOLUME: 80
(E) ISSUE:
(F) PAGES: 3812-3815
(G) DATE: 1983

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: synthetic

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Saiki, R., et al.
(B) TITLE:
(C) JOURNAL: Science
(D) VOLUME: 239
(E) ISSUE:
(F) PAGES: 487
(G) DATE: 1988

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GAAGAGCCAA GGACAGGTAC 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: synthetic

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Saiki, R., et al.
(B) TITLE:
(C) JOURNAL: Science
(D) VOLUME: 239
(E) ISSUE:
(F) PAGES: 487
(G) DATE: 1988

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAACTTCATC CACGTTCACC 20

WHAT IS CLAIMED IS:

1. A method for isolating total nucleic acid having substantially intact RNA from a biological sample comprising RNA, DNA and protein, said method comprising the steps of:

a) incubating said sample in a lysing buffer comprising an ionic detergent, said lysing buffer being substantially free of guanidine compounds, said incubating occurring for a time from less than about 1 minute to about 120 minutes;

b) precipitating a major portion of said protein in said incubated sample by adding a salt composition under conditions wherein said nucleic acid remains substantially in a nucleic acid solution;

c) separating said precipitated protein from said nucleic acid solution;

d) precipitating said nucleic acid from said nucleic acid solution using a nucleic acid-precipitating agent; and

e) recovering said precipitated nucleic acid.

2. The method of claim 1, wherein said lysing buffer further comprises a proteinase.

3. The method of claim 1, wherein said lysing buffer further comprises a ribonuclease inhibitor.

4. The method of claim 3, wherein said ribonuclease inhibitor comprises vanadyl ribonuclease complex.

5. The method of claim 3, wherein said incubating step occurs for a time from less than about 1 minute to about 5 minutes.

6. The method of claim 1, further comprising the step of extracting residual protein from said nucleic acid solution using a protein-extracting agent, said extracting step occurring after said separating step.

7. The method of claim 1, wherein said incubating step occurs at a temperature from about 22°C to about 65°C.

8. The method of claim 1, wherein said recovered nucleic acid is effective as a template in an amplification reaction.

9. The method of claim 8, wherein said recovered nucleic acid comprises RNA effective as a template for amplification by self-sustained sequence replication.

10. The method of claim 8, wherein said recovered nucleic acid comprises RNA effective as a template for amplification by nucleic acid sequence-based amplification.

11. The method of claim 8, wherein said recovered nucleic acid comprises DNA effective as a template for amplification by polymerase chain reaction.

12. An article of manufacture, comprising:

a) packaging material;

b) a lysing buffer within said packaging material, said lysing buffer comprising an ionic detergent, said lysing buffer being substantially free of guanidine compounds; and

c) a label or package insert accompanying said packaging material, said label or package insert indicating that said lysing buffer is suitable for use in a method for isolating total nucleic acid having substantially intact RNA from a biological sample comprising RNA, DNA and protein, said method comprising the steps of:

i) incubating said sample in said lysing buffer for a time from less than about 1 minute to about 120 minutes;

ii) precipitating a major portion of said protein in said incubated sample by adding a salt composition under conditions wherein said nucleic acid remains substantially in a nucleic acid solution;

iii) separating said precipitated protein from said nucleic acid solution;

iv) precipitating said nucleic acid from said nucleic acid solution using a nucleic acid-precipitating agent; and

v) recovering said precipitated nucleic acid.

13. An article of manufacture as recited in claim 12, further comprising a salt composition, said label or package insert further indicating said salt composition is suitable for use in said nucleic acid isolation method.

FIG. 1

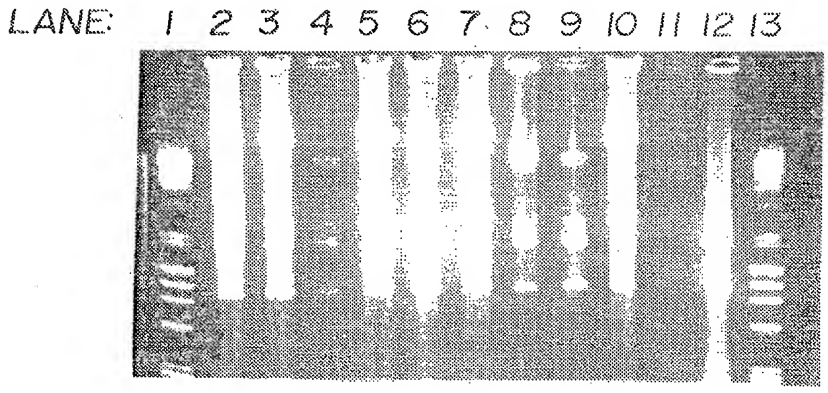


FIG. 3

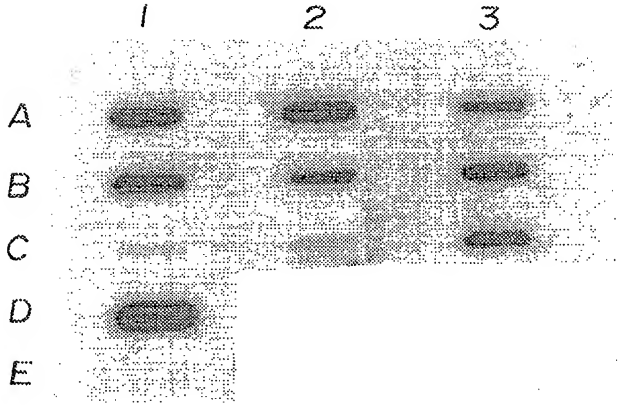


FIG. 2

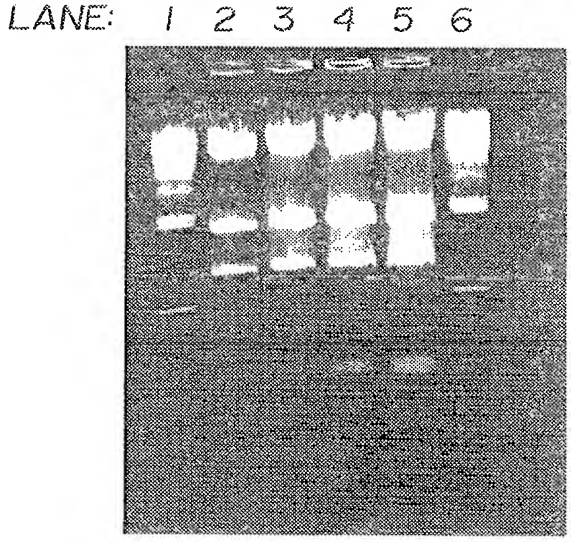
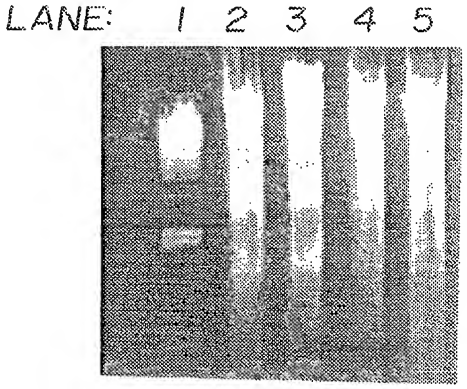


FIG. 4



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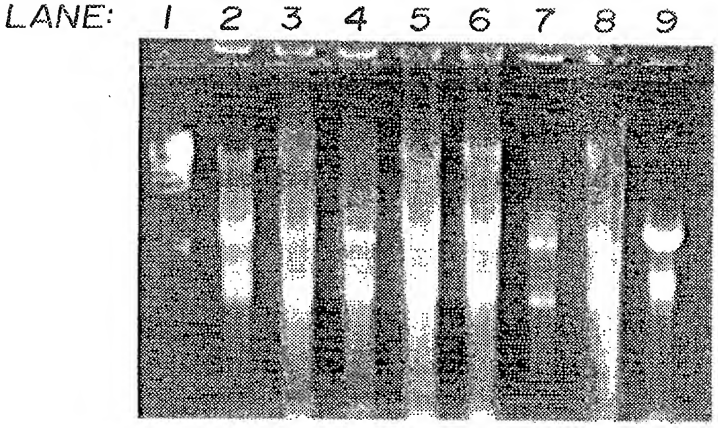

FIG. 6

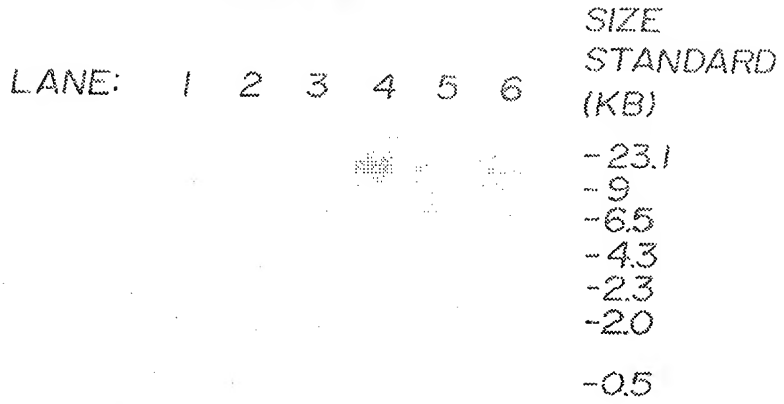
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FIG. 7

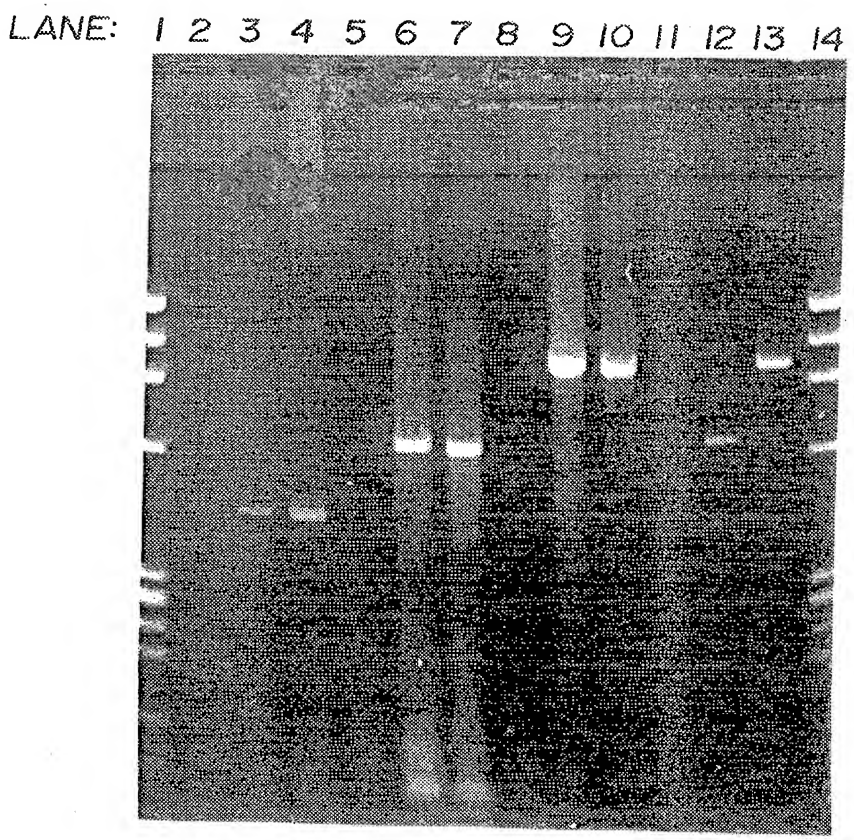
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FIG. 8

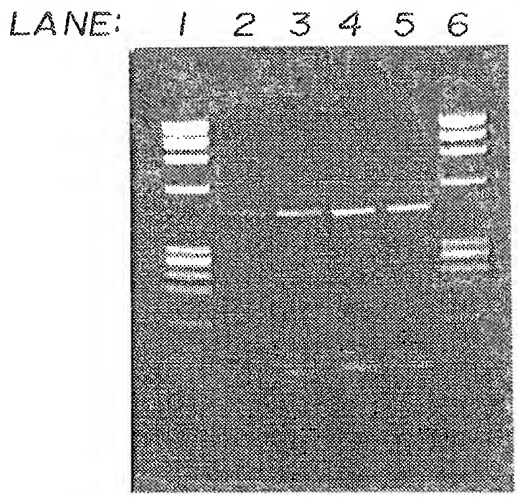
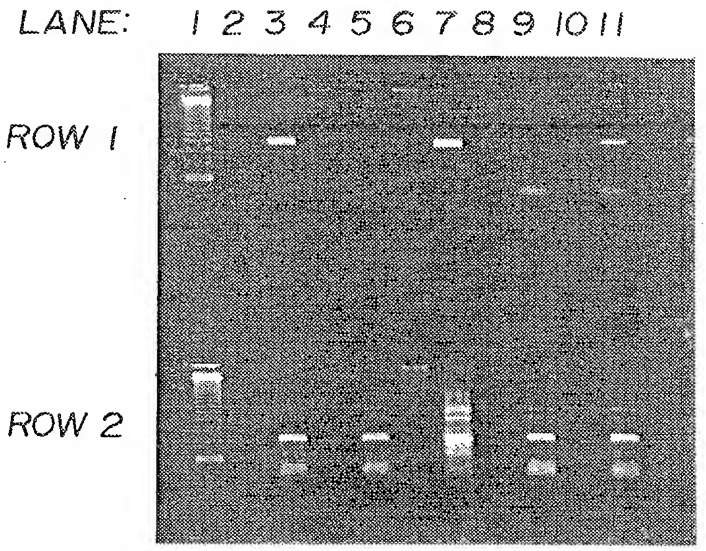


FIG. 9



INTERNATIONAL SEARCH REPORT

Intern. al Application No
PCT/US 95/07940

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H1/08 C12Q1/68 C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,89 07603 (MEMORIAL BLOOD CENTER OF MINNEAPOLIS) 24 August 1989 see the whole document ---	1-6,12, 13
Y	US,A,4 833 239 (DEBONVILLE) 23 May 1989 see the whole document ---	1-13
Y	WO,A,91 18996 (AUTOGEN INSTRUMENTS INC) 12 December 1991 see the whole document ---	1-13
Y	WO,A,94 12657 (KATCHER) 9 June 1994 page 10 - page 11, line26; claims 1-29 see page 1 - page 4, line 14 ---	1-13
Y	WO,A,90 02179 (LIFECODES CORP) 8 March 1990 see page 1 - page 2, line 24 ---	1-13

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[X] Further documents are listed in the continuation of box C.

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4 October 1995	17. 10. 95

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO,A,92 00983 (MICROPROBE CORP) 23 January 1992 see page 1 - page 4, line 15	1-13

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